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(54) Title: HELICOBACTER PYLORI LIVE VACCINE

(57) Abstract

The present invention relates to novel recombinant live vaccines, which provide protective immunity against an infection by Helicobacter pylori and a method of screening H. pylori antigens for optimized vaccines.

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Helicobacter pylori live vaccine

Specification

The present invention relates to novel recombinant live vaccines, which provide protective immunity against an infection by Helicobacter pylori and a method of screening H. pylori antigens for optimized vaccines.

Helicobacter is a gram-negative bacterial pathogen associated with the development of gastritis, pepic ulceration and gastric carcinoma. Several Helicobacter species colonize the stomach, most notably H. pylori, H. heilmanii and H. felis. Although H. pylori is the species most commonly associated with human infection, H. heilmanii and H. felis also have been found to infect humans. High H. pylori infection rates are observed in third world countries, as well as in industrialized countries. Among all the virulence factors described in H. pylori, urease is known to be essential for colonisation of gnobiotic pigs and nude mice. Urease is an enzyme composed of two structural subunits (UreA and UreB). Previous studies have indicated that oral immunization using recombinant UreB plus cholera toxin were able to protect mice from gastric colonisation with H. felis and H. pylori (Michetti et al., Gastroenterology 107 (1994), 1002-1011). By oral administration of recombinant UreB antigens, however, in several cases only an incomplete protection can be obtained. Other H. pylori antigens shown to give partial protection are the 87 kD vacuolar cytotoxin VacA (Cover and Blaser, J. Biol. Chem. 267 (1992), 10570; Marchetti et al., Science 267 (1995), 1655) and the 13 and 58 kD heat shock proteins HspA and HspB (Ferrero et al., Proc. Natl. Acad. Sci. USA 92 (1995), 6499).

Attenuated pathogens, e.g. bacteria, such as Salmonella, are known to be efficient live vaccines. The first indications of the efficacy of attenuated Salmonella as good vaccine in hu-

mans came from studies using a chemically mutagenized Salmonella typhi Ty21a strain (Germanier and Furer, J. Infect. Dis. 141 (1975), 553-558), tested successfully in adult volunteers (Gilman et al., J. Infect. Dis. 136 (1977), 717-723) and later on in children in a large field trial in Egypt (Whadan et al., J. Infect. Dis. 145 (1982), 292-295). The orally administered Ty21a vaccine was able to protect 96% of the Egyptian children vaccinated during three years of surveillance. Since that time new attenuated Salomonella live vector vaccines have developed (Hone et al., Vaccine 9 (1991), 810-816), in which well defined mutations incorporated into the chromosome gave rise to non-virulent strains able to induce strong immune responses after oral administration (Tacket et al., Vaccine 10 (1992), 443-446 and Tacket et al., Infect. Immun. 60 (1992), 536-541). Other advantages of the live attenuated Salmonella vaccine include its safety, easy administration, long-time protection and no adverse reactions in comparison with the former inactivated wholesale typhoid vaccines (Levine et al., Typhoid Fever Vaccines. In: Plotkin S.A., Mortimer E.A. Jr. (eds.) Vaccines. Philadelphia: WB Saunders (1988), 333-361).

Mutants of S. typhimurium have been extensively used to deliver antigens because of the possibility to use mice as an animal model, which is believed to mimick S. typhi infections in humans. The attenuation of S. typhimurium most commonly used consists in site directed mutagenesis of genes affecting the synthesis of aromatic amino acids. Such strains, designated aro mutants, have a negligible pathogenicity, as demonstrated in animal models and human trials using these constructs (Hoiseth and Stocker, Nature 291 (1981), 238-239; Tacket et al. (1992), Supra). Advantage has been taken from the potent immunogenicity of live Salmonella vaccine to deliver heterologous antigens. Expression of specific antigens in attenuated Salmonella has conferred murine protection against several bacterial pathogens. The use of recombinant live vaccines, which are capable of expressing Helicobacter antigens and protecting the vaccinated animals, has not yet been described.

The use of attenuated live vaccines for the treatment of a Helicobacter infection has also not been rendered obvious. The reason therefor being that in the course of the Helicobacter infection a strong immune response against the pathogen per se is induced, which, however, does not lead to a protective immunity. Thus, it was highly surprising that a protective immune response is achieved when using recombinant attenuated bacterial cells as antigen carriers, which are capable of expressing a DNA molecule encoding a Helicobacter antigen. Apparently, recombinant attenuated bacterial cells expressing a Helicobacter antigen are capable of creating a qualitatively different immune response against the heterologous Helicobacter antigen than Helicobacter itself does against its own homologous antigen. Surprisingly, a non-protective immune response is thus transformed into an immune response protecting against Helicobacter infections. This unexpected observation renders it possible to use recombinant attenuated pathogens, e.g. bacterial cells, particularly Salmonella, as carriers for the screening of protective antigens, to apply the protective antigens identified in this manner in any vaccine against Helicobacter infections, and to use recombinant attenuated bacteria as carriers of protective antigens for the immunization against Helicobacter infections in humans and other mammals.

Thus, a subject matter of the present invention is a recombinant attenuated pathogen, which comprises at least one heterologous nucleic acid molecule encoding a Helicobacter antigen, wherein said pathogen is capable to express said nucleic acid molecule or capable to cause the expression of said nucleic acid in a target cell. Preferably the nucleic acid molecule is a DNA molecule.

The attenuated pathogen is a microorganism strain which is able to cause infection and preferably effective immunological protection against the actual pathogen but is no longer pathogenic per se. The attenuated pathogen can be a bacterium, a

virus, a fungus or a parasite. Preferably it is a bacterium, e.g. Salmonella, such as S. typhimurium or S. typhi, Vibrio cholerae (Mekalanos et al., Nature 306 (1983), 551-557), Shigella Species such as S. flexneri (Sizemore et al., Science 270 (1995), 299-302; Mounier et al., EMBO J. 11 (1992), 1991-1999), Listeria such as L. monocytogenes (Milon and Cossart, Trends in Microbiology 3 (1995), 451-453), Escherichia coli, Streptococcus, such as S. gordonii (Medaglini et al., Proc. Natl. Acad. Sci. USA 92 (1995) 6868-6872) or Mycobacterium, such as Bacille Calmette Guerin (Flynn, Cell. Mol. Biol. 40 Suppl. 1 (1994), 31-36). More preferably the pathogen is an attenuated enterobacterium such as Vibrio cholerae, Shigella flexneri, Escherichia coli or Salmonella. Most preferably the attenuated pathogen is a Salmonella cell, e.g. a Salmonella aro mutant cell. The attenuated pathogen, however, can be a virus, e.g. an attenuated vaccinia virus, adenovirus or pox virus.

The nucleic acid molecule which is inserted into the pathogen codes for a Helicobacter antigen, preferably a H. felis, H. heilmanii or H. pylori antigen, more preferably a H. pylori antigen. The Helicobacter antigen can be a native Helicobacter polypeptide, an immunologically reactive fragment thereof, or an immunologically reactive variant of a native polypeptide or of a fragment thereof. Further, the Helicobacter antigen can be a protective carbohydrate or a peptide mimotope simulating the three-dimensional structure of a native Helicobacter antigen. Peptide mimotopes can be obtained from peptide libraries presented on the surface of bacterial cells (cf. PCT/EP96/01130). Of course, the transformed cell can also contain several DNA molecules coding for different Helicobacter antigens.

The nucleic acid molecules coding for Heliobacter antigens may be located on an extrachromosomal vector, e.g. a plasmid, and/or integrated in the cellular chromosome of the pathogen. When the pathogen is used as a vaccine, chromosomal integra-

tion usually is preferred.

Attenuated bacteria can be used to transcribe and translate said nucleic acid molecule directly in the bacterial cell or to deliver said nucleic acid molecule to the infected target cell, such that the DNA molecule is transcribed and/or translated by the eukaryotic target cell machinery. This indirect bacterial vaccination procedure, termed here as genetic vaccination, has been successfully used with Shigella as a carrier (Sizemore, D. R., Branstrom, A. A. & Sadoff, J. C. (1995) Attenuated Shigella as a DNA delivery vehicle for DNA-mediated immunization. Science 270:299-302).

In a preferred embodiment of the present invention the Helicobacter antigen is urease, a urease subunit or an immunologically reactive variant or fragment thereof or a peptide mimotope thereof. In a further preferred embodiment of the present invention the Helicobacter antigen is a secretory polypeptide from Helicobacter, an immunologically reactive variant or fragment thereof or a peptide mimotope thereof. A process for identifying Helicobacter genes coding for such secretory polypeptides, and particularly for adhesins, has been disclosed in the international patent application PCT/EP96/02544, which is incorporated herein by reference. This process comprises

a) preparing a gene bank of H. pylori DNA in a host organism containing an inducible transposon coupled to a marker of secretory activity,

b) inducing the insertion of the transposon into the H. pylori DNA and

c) conducting a selection for clones containing a secretory gene by means of the marker, and optionally further

d) conducting a retransformation of H. pylori by means of the DNA of clones containing genes having secretory activity, wherein isogenic H. pylori mutant strains are produced by means of integrating the DNA into the chromosome, and

e) conducting a selection detecting adherence-deficient H. pylori mutant strains.

Suitable examples of antigens obtainable by the above process are selected from the group consisting of the antigens AlpA, AlpB, immunologically reactive variants or fragments thereof or peptide mimotopes thereof. The nucleic and amino acid sequences of the antigens AlpA and AlpB have been disclosed in the international patent applications PCT/EP96/02545 and PCT/-EP96/04124, which are incorporated herein by reference. Further, the nucleic and amino acid sequences of AlpB are shown in SEQ ID NO. 1 and 2, and the nucleic and amino acid sequences of AlpA in SEQ ID NO. 3 and 4.

It is also conceivable, however, that an intracellular antigen is used which can be presented on the surface, e.g. by autolytic release, and confers immunological protection.

The presentation of the Helicobacter antigens in the recombinant pathogen according to the invention can be accomplished in different ways. The antigen or the antigens can be synthesized in a constitutive, inducible or phase variable manner in the recombinant pathogen. Concerning the constitutive or inducible synthesis of the Helicobacter antigens known expression systems can be referred to, as have been described by Sambrook et al., Molecular Cloning, A Laboratory Manual (1989), Cold Spring Harbor Laboratory Press.

Particularly preferred the antigens are presented in a phase variable expression system. Such a phase variable expression system for the production and presentation of foreign antigens in hybrid live vaccines is disclosed in EP-B-0 565 548, which is herein incorporated by reference. In such a phase variable expression system the nucleic acid molecule encoding the Helicobacter antigen is under control of an expression signal, which is substantially inactive in the pathogen, and which is capable of being activated by a spontaneous reorganization

caused by a nucleic acid, e.g. DNA reorganization mechanism in the pathogen, e.g. a specific DNA inversion process, a specific DNA deletion process, a specific DNA replication process or a specific slipped-strand-mispairing mechanism.

A recombinant cell having a phase variable expression system is capable of forming two subpopulations A and B, wherein the division into said subpopulations occurs by spontaneous reorganization in the recombinant nucleic acid, wherein said subpopulation A is capable of infection and immunologically active per se, while subpopulation B, which is regenerated from subpopulation A, produces at least one heterologous Helicobacter antigen and acts immunologically with respect to said additional antigen.

The activation of the expression signal encoding the Helicobacter antigen can be directly accomplished by nucleic acid reorganization or, alternatively, indirectly accomplished by activation of a gene encoding a protein which controls the expression of the gene encoding the Helicobacter antigen. The indirect activation represents a system which allows the production of the Helicobacter antigen via a cascade system, which can be realized e.g. in that the gene directly controlled by DNA reorganization codes for an RNA polymerase which is specific for the promoter preceding the Helicobacter gene, or a gene regulator which in another specific manner induces the expression of the Helicobacter gene. In an especially preferred embodiment of the present invention the expression signal for the gene encoding the Helicobacter antigen is a bacteriophage promoter, e.g. a T3, T7 or SP6 promoter, and the activation of the expression signal is caused by a nucleic acid reorganization resulting in the production of a corresponding bacteriophage RNA polymerase in the pathogen.

The phase variable expression system can be adjusted to provide a preselected expression level of the Helicobacter antigen. This can be accomplished e.g. by modifying the nucleotide

sequence of the expression signal, which is activated by the nucleic acid reorganization mechanism, and/or by inserting further genetic regulation elements.

The Helicobacter antigens can be produced in an intracellular, as well as in an extracellular manner in the pathogen according to the invention. For instance, autotransporter systems such as the IgA-protease system (cf. for instance EP-A-0 254 090) or the E. coli AIDA-1 adhesin system (Benz et al., Mol. Microbiol. 6 (1992), 1539) are suited as extracellular secretory system. Other suitable outer membrane transporter systems are the RTX-toxin transporters, e.g. the E. coli hemolysin transport system (Hess et al., Proc. Natl. Acad. Sci. USA 93 (1996), 11458-11463).

The pathogen according to the invention can contain a second heterologous nucleic acid, e.g. DNA molecule, which codes for an immunomodulatory polypeptide influencing the immune response quantitatively or qualitatively, apart from the nucleic acid molecule encoding the Helicobacter antigen. Examples of such immunomodulatory polypeptides are immune-stimulating peptides, cytokines like IL-2, IL-6 or IL-12, chemokines, toxins, such as cholera toxin B or adhesins.

The present invention also refers to a pharmaceutical composition comprising as an active agent a recombinant attenuated pathogen as described above, optionally together with pharmaceutically acceptable diluents, carriers and adjuvants. Preferably, the composition is a living vaccine. The vaccination routes depend upon the choice of the vaccination vector. The administration may be achieved in a single dose or repeated at intervals. The appropriate dosage depends on various parameters such as the vaccinal vector itself, or the route of administration. Usually the dosage comprises about 10^6 to 10^{12} cells (CFU), preferably about 10^8 to 10^{10} cells (CFU) per vaccination. Administration to a mucosal surface (e.g. ocular, intranasal, oral, gastric, intestinal, rectal, vaginal or urinary tract)

or via the parenteral route (e.g. subcutaneous, intradermal, intramuscular, intravenous or intraperitoneal) might be chosen. A method for the preparation of the living vaccine comprises formulating the attenuated pathogen in a pharmaceutically effective amount with pharmaceutically acceptable diluents, carriers and/or adjuvants.

The pharmaceutical composition may be provided in any suitable form, e.g. a suspension in suitable liquid carrier, such as water or milk, a capsule, a tablet etc. In a preferred embodiment of the present invention the composition is a lyophilized product which is suspended in a liquid carrier prior to use.

Further, the present invention refers to a method for preparing a recombinant attenuated pathogen as defined above, comprising the steps of a) inserting a nucleic acid molecule encoding a Helicobacter antigen into an attenuated pathogen, wherein the recombinant pathogen, e.g. a transformed bacterial cell, is obtained, which is capable of expressing said nucleic acid molecule or is capable to cause expression of said nucleic acid molecule in a target cell and b) cultivating said recombinant attenuated pathogen under suitable conditions. If the pathogen is a bacterial cell, the nucleic acid molecule encoding the Helicobacter antigen can be located on an extrachromosomal plasmid. It is, however, also possible to insert the nucleic acid molecule into the chromosome of the pathogen.

Furthermore, the present invention refers to a method for identifying Helicobacter antigens which raise a protective immune response in a mammalian host, comprising the steps of: a) providing an expression gene bank of Helicobacter in an attenuated pathogen and b) screening the clones of the gene bank for the ability to confer a protective immunity against a Helicobacter infection in a mammalian host. Preferably, this identification process takes place in a phase variable expression system, rendering possible a stable expression of all of

the Helicobacter antigens. Recombinant clones can then be applied as "pools" for the oral immunization of test animals, such as mice. The potential of these clones as protective antigen is then determined via a challenge infection with Helicobacter, e.g. a mouse-adapted H. pylori strain. Thus, there is a possibility of directly selecting optimized H. pylori vaccine antigens.

The invention will be further illustrated by the following figures and sequence listings.

Fig. 1: shows a schematic illustration of the urease expression vector pYZ97, whereon the genes coding for the urease subunits UreA and UreB are located under transcriptional control of the T7 promoter ϕ10. There is a ribosomal binding site (RBS) between the T7 promoter and the urease genes. Further, the plasmid exhibits an origin of replication (ori), a ß-lactamase resistance gene (bla) and 4 T7 terminators in series.

Apart from the expression by the T7 promoter, a constitutive low level expression of the urease A and B subunits can also be brought about via a cryptic promoter, which is located upstream from the T7 promoter, on the plasmid pYZ97.

Fig.2: shows the nucleotide sequence of the transcriptional regulation region for urease expression and the beginning of the amino acid sequence of urease subunit A on plasmid pYZ97.

Fig.3: shows a schematic illustration of the T7 RNA polymerase (T7RNAP) expression cassettes pYZ88, pYZ84 and pYZ114, which can be integrated into the chromosomes of bacteria.

In the high-expression cassette pYZ88 the lambda PL promoter is located in inverse orientation, upstream from the T7RNAP gene. A gene for the temperature-sensitive repressor cI 857 (cI) is under control of this promoter. A terminator of the bacteriophage fd (fdT) is situated upstream from the cI gene. The gin gene (Mertens, EMBO J. 3 (1984), 2415-2421) codes for a control enzyme of a DNA reorganization mechanism. A DNA sequence coding for the tRNA Arg is located downstream from the gin gene.

In phase A the PL promoter responsible for the expression of the T7RNAP gene is directed in the direction of the cI857 gene and the gin gene. The consequence of this is that an active repressor is formed at the permissive temperature of 28°C and reduces the transcription from the PL promoter. At a higher temperature the transcription of the PL promoter is increased, since the repressor is inactivated at least partially under such external influences. The temperature-dependent increase in the transcription also causes a corresponding increase in the expression of the following gin gene, which as a control enzyme catalyses the inversion of the PL promoter and the transition in phase B, in which the T7RNAP gene is expressed.

In the high-expression system pYZ88 a further fdT transcription terminator is located between a kanamycin-resistance gene (km) and the promoter of this gene. In this manner, the synthesis of an anti-sense RNA, inversely orientated to the T7RNAP gene, which normally contributes to the reduction of the T7RNAP expression, is reduced. This results in a high expression of the T7RNAP.

In the medium-expression system pYZ84 a transcrip-

tion terminator (fdT) is located between the PL promoter and the start of the T7RNAP gene. In this manner the expression of the T7RNAP mRNA is reduced. Additionally, the anti-sense RNA affects the T7RNAP translation. Therefore, only a medium expression occurs.

In the low-expression system pYZ114 a deletion of 100 bp in PL is additionally introduced (Δ PL). In this manner the activity of the PL promoter is reduced to a high extent, which leads to a lower T7RNAP expression and thus to a reduction of the UreA/B gene expression. In this construct the effect of the cryptic promoter on pYZ97 is already observed.

Fig.4: shows the results of an ELISA for anti-H.pylori antibodies in intestinal fluids of vaccinated mice.

Fig.5: shows the results of an ELISA for anti-H.pylori antibodies in the serum of vaccinated mice.

Fig.6: shows the urease activity in the stomach tissue of vaccinated mice after H.pyroli challenge.

SEQ ID NO. 1 and 2 show the nucleotide sequence of the adhesin gene AlpB from H. pylori and the amino acid sequence of the polypeptide coded therefrom.

SEQ ID N0. 3 and 4 show the nucleotide sequence of the adhesin gene AlpA from H. pylori and the amino acid sequence of the protein coded therefrom.

SEQ ID NO. 5 and 6 show the nucleotide sequence of the transcriptional regulation region for urease expression and the beginning of the amino acid sequence of urease subunit A on plasmid pYZ97.

Experimental part

Example 1

Cloning of the *ureA* and *ureB* genes.

The structural genes encoding the urease, *ureA* and *ureB*, have been genetically cloned from chromosomal DNA of a clinical specimen P1 (formerly 69A) isolated at the University of Amsterdam and provided by Dr. Jos van Putten. The genes were isolated by a PCR-approach using the primer pair YZ019 (5´-GGAATTCCATATGAAACTGACTCCCAAAGAG-3´) and RH132 (5´-CTGCAGTCGACTAGAAAATGCTAAAGAG-3´) for amplification. The sequence of the primers was deduced from GenBank (accession numbers M60398, X57132). The DNA sequence of primer YZ019 covered the nucleotides 2659-2679 of the published sequence and further contained a translational regulatory sequence (down stream box; Sprengart, M. L. et al., 1990, Nuc. Acid. Res. 18:1719-1723) and a cleavage site for *NdeI*. The DNA sequence of primer RH132 covered the nucleotides 5071-5088 of the published sequence and a cleavage site for *SalI*. The amplification product was 2.4 kbp in size comprising the complete coding region of *ureA* and *ureB* genes without the original transcriptional start and termination sequences from the *Helicobacter* chromosome. The purified PCR-fragment was digested with *NdeI* and *SalI* and inserted into the corresponding cloning sites of T7 expression plasmid pYZ57 to yield the plasmid pYZ97.

pYZ57 was originally derived from plasmid pT7-7, which was described by Tabor (1990, *In* Current Protocols in Molecular Biology, 16.2.1-16.2.11. Greene Publishing and Wiley-Interscience, New York). Two terminator fragments were introduced into the pT7-7 backbone at different sites by the following strategy: (1) The tandem T7 terminators. A 2.2 kbp *EcoRI/HindIII* fragment was excised from pEP12 (Brunschwig & Darzins, 1992, Gene, 111:35-41) and the purified fragment ligated with predigested pBA (Mauer, J. et al., 1997, J. Bacteriol. 179:794-804). The ligation product was digested

with *HindIII* and *ClaI*. The resulting 2.2 kbp *HindIII/ClaI* fragment was subsequently inserted into predigested pT7-7. (2) The T1 terminator. A 230 bp *HpaI/NdeI*-fragment was excised from plasmid pDS3EcoRV (provided by Dr. H. Bujard; ZMBH, Heidelberg). The fragment was then further treated with *Klenow* to generate blunt ends. The purified rrnBT1 fragment was inserted into the previous pT7-7 derivative, predigested with *BglII* and subsequently bluntended by Klenow treatment. Figure 1 describes the completed vector pYZ97 used for the expression of the urease genes coding for urease subunits UreA and UreB in *S. typhimurium*. As indicated in Figure 1, the urease genes can be controlled by the T7 promoter ϕ10. The ribosome binding site (RBS) is located between the T7 promoter and the urease genes. Further, the plasmid exhibits an origin of replication (*ori*) and a ß-lactamase resistance gene (*bla*).

Apart from the expression controled by the T7 promoter, a constitutive moderate level expression of the urease A and B subunits does occur from a promoter driven by *Salmonella* RNA polymerase. The promoter is located upstream from the T7 promoter, on the plasmid pYZ97. For detailed molecular analysis, the purified *BglII/HindIII*-fragment of pYZ97 was subcloned into the pCR-Script™ SK(+)kit (Stratagene) and subjected to DNA-sequencing. The sequence data confirmed the various elements in their completeness (see Figure 2 and SEQ ID NO.5 and 6): part of the *ureA* gene, the down-stream box, the RBS, the T7 promoter and the T1 terminator (rrnBT1). The sequence analysis also disclosed the region between the T1 terminator region and the T7 promoter where the *Salmonella* RNA polymerase promoter is localised. The sequence data suggests a location of this constitutive promoter between nucleotides 222 - 245 which have been deduced from structural predictions (Lisser & Margalit, 1993, Nuc. Acid. Res. 21:1507-1516).

Example 2

Immunological protection by administration of live vaccine

Materials and Methods

Bacterial strains: S. typhimurium SL3261 live vector vaccine strain was used as a recipient for the recombinant H. pylori urease plasmid constructs. S. typhimurium SL3261 is an aroA transposon mutant derived from S. typhimurium SL1344 wild type strain. S. typhimurium SL3261 is a non-virulent strain that gives protection to mice against infection with wild type S. typhimurium after oral administration (Hoiseth and Stocker (1981) Supra). S. typhimurium SL3261 and derivatives thereof, which contain the urease expression plasmid pYZ97 (extrachromosomal) and the T7RNAP expression cassettes pYZ88, pYZ84 or pYZ114, respectively (integrated into the chromosome) are indicated in table 1. Luria broth or agar was used for bacterial growth at 28°C. H. pylori wild type strain grown at 37°C on serum plates was used for the challenge experiments.

Immunization of mice: Four weeks Balb/c mice purchased from Interfauna (Tuttlingen, Germany) were adapted two weeks in an animal facility before being used for experimentation. 150 µl of blood was taken retroorbitally from all mice to obtain preimmune serum. Retroorbital bleedings were repeated from all immunized mice 1 week and 3 weeks after immunization.

Eight groups of 5 mice including controls were used in this study (table 2). Group A, the naive control group, was not immunized with Salmonella neither challenged with wild type H. pylori. The rest of the groups were all orally immunized. Group B, a negative control group, did not receive Salmonella and was challenged with H. pylori. Mice from groups C to G were immunized with Salmonella vaccine strains and challenged with H. pylori. The last group H received recombinant urease B in combination with cholera toxin and was also challenged.

Prior to immunizations mice were left overnight without solid food and 4 hours without water. 100 µl of 3% sodium bicarbonate were given orally using a stainless steel catheter tube to neutralize the stomach pH. Then mice from group B received 100 µl PBS and mice from groups C to G received 1.0×10^{10} CFU

of Salmonella in a 100 µl volume. Mice from group H received four times 100 µl of a mixture of recombinant H. pylori UreaseB plus cholera toxin, one dose every week. After every immunization water and food were returned to the mice.

H. pylori challenge: Four weeks after the first oral immunization mice from groups B to H were challenged with H.pylori. Mice were left overnight without solid food and without water 4 hours prior to the challenge. 100 µl of 3% sodium bicarbonate were given orally to the mice using a stainless steel catheter tube, followed by an oral dose of 5.0 x 10^9 CFU/ml of Helicobacter pylori. Water and food were returned to the mice after the challenge.

Collection of blood and tissues from mice: Twelve weeks after the first immunization the mice were left overnight without food and subsequently sacrificed for analysis of protection and immune response. The mice were anaesthetized with Metoxyfluorane for terminal cardiac bleeding and prior to sacrifice by cervical dislocation. Under aseptic conditions, spleen and stomach were carefully removed from each mouse and placed on ice in separate sterile containers until further processing. Large and small intestine were obtained for further isolation of the intestinal fluid.

Processing of stomach and measurement of urease activity: The degree of H. pylori colonisation in the mouse stomach was measured by the presence of active urease in the tissue. The Jatrox-test (Röhm-Pharma GmbH, Weiterstadt, Germany) was used according to the suppliers' directions. Stomach mucosa was exposed and washed with PBS, half of the antral portion of the stomach was immediately placed inside an Eppendorf tube containing the substrate for measurement of urease activity. Absorbance at 550 nm was measured after tubes were incubated for 4 hours at room temperature. The rest of the stomach tissue was stored at -20°C for further treatments. The urease activity values obtained from the stomach of naive mice, which

did not undergo immunization or challenge, were used to create a base line to indicate the absence of H. pylori infection and therefore protection.

Table 1

UreA and UreB expressing S. typhimurium vaccine strains

Strains	Urease Expression	Source
S. typhimurium SL3261	Negative	Hoiseth and Stocker
S. typhimurium SL3262 pYZ97	Constitutive Low	this study
S. typhimurium SL3261::pYZ88pYZ97	High T7-induced expression	this study
S. typhimurium SL3261::pYZ84pYZ97	Medium T7-induced expression	this study
S. typhimurium SL3261::pYZ114pYZ97	Low T7-induced expression	this study

Table 2

Mice groups used for immunization

Group	Immunogen	No. of oral immuniza-tions
A	None	0
B	PBS oral immunization	1
C	S. typhimurium S3261	1
D	S. typhimurium S3261 pYZ97	1
E	S. typhimurium S3261::pYZ88pYZ97	1
F	S. typhimurium S3261::pYZ84pYZ97	1
G	S. typhimurium S3261::pYZ114pYZ97	1
H	Urease B plus cholera toxin	4

Results:

In the control mice (groups B and C) 100% infection with H. pylori was observed. In the mice immunized with recombinant attenuated pathogens (groups D, E, F, G) between 0% and 60% infection (100% to 40% protection) was observed. Immuno-protection did not correlate with humoral anti-UreA and UreB response, suggesting that, in addition to humoral immunity, cellular immunity is critical for protection against H. pylori infection. The results indicate that oral immunization of mice with UreA and UreB delivered by S. typhimurium attenuated strain is effective to induce high levels of protection against H. pylori colonisation.

In the mice immunized with recombinant urease B plus cholera toxin considerably higher levels of urease activity were ob-